Enhancing crop productivity by CRISPR-mediated genetic improvement of root architecture: a focus on phytohormones

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Abstract

Food security is one of the main topics of today's agriculture especially facing challenging environmental conditions. As most humankind has a daily intake of cereal grains, current breeding programs focus on these crop plants. Within the breeders' toolbox, customised endonucleases became included after this universal application had been demonstrated. Due to technological restrictions, the main focus was on aboveground plant organs, while the essential belowground has been given only limited attention. In the present review, we summarise the knowledge on the root system architecture in cereals, the importance of phytohormones in this physiological process, and the molecular mechanisms involved. The review summarises how the use of the CRISPR methodology can improve the root system architecture to enhance crop production genetically. Finally, future research directions involving all this knowledge and technical advances are suggested.

Enhancing crop productivity by CRISPR/Cas-mediated genetic improvement of root architecture: a focus on phytohormones

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1. Abstract

Food security is one of the main topics of today's agriculture especially facing challenging environmental conditions. As most humankind has a daily intake of cereal grains, current breeding programs focus on these crop plants. Within the breeders' toolbox, customised endonucleases became included after this universal application had been demonstrated. Due to technological restrictions, the main focus was on aboveground plant organs, while the essential belowground has been given only limited attention. In the present review, we summarise the knowledge on the root system architecture in cereals, the importance of phytohormones in this physiological process, and the molecular mechanisms involved. The review summarises how the use of the CRISPR methodology can improve the root system architecture to enhance crop production genetically. Finally, future research directions involving all this knowledge and technical advances are suggested.

2. Introduction

Grain cereals are an essential food supply, representing more than 78% of daily human calories (FAO Stat). The use of grains as a food source began already during the Middle Stone Age, long before cereal domestication. With the growing world population, the global food demand is also increasing. In the present context of climate change and intensive agriculture that leads to soil degradation, higher desertification and salinization of cultivated areas are expected in the very near future. In this regard, the sustainability of cereal yield has become a challenge for food security. To safeguard environmental quality, usage of fertilizers and watering has to be reduced. One has to exploit the intrinsic ability of plants to respond and adapt to adverse conditions, notably to a temporary period of drought/flooding or suboptimal nutrient supplies. Historically, research in the field has focused on the aboveground plant parts, neglecting the underground organs. This hidden status renders difficult access to intact root systems for analysis, particularly field experimentation. In the last decades, methods to study roots have evolved from destructive to non-destructive imaging techniques.

Roots play critical plant functions, serving not only for anchorage but also for water and nutrient uptake and transport, storage, communication and interaction with the soil microbiome and other plants. The root system architecture (RSA) describes the 3D organization of the roots in the soil and refers to root morphology, topology and distribution. The RSA is shaped by the combination of the genetic background, the availability of water and nutrients whose distribution is heterogeneous in the soil, and the stress response. Among nutrients, nitrate (N) and phosphorus (P) probably have the highest impact on plant growth and crop yield. Whereas N is highly soluble and will leak in the deep soil strata, P is quickly immobilized in the surface soil layers. Therefore, deep rooting will ensure N acquisition from deep soil layers, whereas a shallow RSA enables plants to use P accumulated in the topsoil. RSA reflects the soil volume that plants can explore. Its description includes branch number, branching pattern, length, orientation, angle or deepness, root diameter, and surface area. Several ideotypes have been described in the literature.

In cereal crops, the root system consists of the embryonically formed primary and seminal roots and the post-embryonically developed nodal roots. These latest arise from the consecutive shoot nodes below the ground (referred to as "crown") and are called crown roots. In adult cereal plants, crown roots form the entire rootstock, resulting in the characteristic fibrous root system. In maize, another type of nodal roots develops from aboveground; they are called brace roots and serve as anchorage. Both embryonically and post-embryonically formed roots share the ability to produce highly branched lateral roots.

In recent years, the genetics of RSA has been revealed while using mutants in different crops species affected in their RSA. Using quantitative genetic and transcriptomic approaches, quantitative trait loci (QTL) and genes involved in RSA in crops have been discovered. Although lateral and crown roots have different origins (root-to-root and shoot-to-root, respectively), conserved molecular mechanisms have been described. In rice, a detailed picture of the genes involved in root elongation and lateral and crown root initiation and emergence has appeared in the last few years. Phytohormones have been described for their role in the process, with auxin being the central actor. Approaches to improve cereals' RSA include classical transgenic manipulation, either overexpression or silencing, of genes involved in phytohormone synthesis and signalling. However, pleiotropic effects on the overall plant development were reported. This highlighted the need to

improve the precision of transgene expression. With the new area of CRISPR-mediated gene editing, one can expect that this goal will be reached.

3. Root system architecture in cereals

In cereals, the root system consists of a primary and several seminal roots formed during embryogenesis and post-embryonic lateral and crown roots, representing the root system (Fig 1A). Primary and seminal roots emerge during germination to ensure water and nutrient uptake by the emerging seedling. Short after germination, post-embryonic roots are formed, elaborating sequentially the root system required to exploit limited soil resources and respond to changing environmental conditions. In cereals, primary and seminal roots are either ephemeral and short-lived like in rice or remain functional over the whole life cycle like barley, maize or wheat. The post-embryonic root system is composed of the lateral and crown roots. Crown roots are shoot-borne roots arising from the lower stem nodes of the main shoot and tillers. They are established throughout the development of the plant, resulting finally in the buildup of the majority of the mature root system. Lateral roots are root-borne roots developing from primary, seminal and crown roots; they confer several branching orders of the RSA. Altogether, they contribute to a fibrous root system, typical of the monocots. Up to now, cereals root systems have been studied in different species of cereals, including rice (*Oryza sativa*), maize (*Zea mays* L.), or Pearl millet (*Pennisetum glaucum* (L.) R. Br.).

Plants can sense the environment and nutrients and readjust their root developmental program to optimize soil's foraging. This ability to modulate root growth angle toward a specific resource or environmental stimulus shape the RSA. Studying the molecular regulation of the RSA, which is a crucial determinant for plant anchorage, water and nutrient uptake efficiency, and the establishment of plant microorganisms' communities, promises to improve crop yield.

4. Phytohormones orchestrate cereals' root system architecture

Environmental factors and phytohormone shape cereals' RSA. However, the hormonal interplay regulating RSA is very complex. Figure 1B offers a simplified overview. For further information, one can refer to recent reviews on the topic.

Auxin is the central regulator of the RSA. This is supported by the fact that most of the mutants reported being affected in their RSA are related to auxin synthesis, transport or signalling pathway. In rice, a recent reviewalso highlights auxin's central role in root elongation and lateral and crown roots initiation and emergence. However, other hormones such as cytokinins (CKs), ethylene, abscisic acid (ABA), gibberellins, brassinosteroids and strigolactones should not stay aside.

4.1. Hormones in primary, lateral and crown roots development

Auxin is the leading actor of root development. Whatever the root's type is considered, all always start in the cells that will initiate a root by establishing an auxin maximum via auxin efflux transport PIN FORMED (PIN) proteins, stabilized by the CROWN ROOTLESS4/OsGNOM1. Auxin is perceived by the auxin receptor TRANSPORT INHIBITOR RESPONSE 1(TIR1)/AUXIN SIGNALING F-BOX (AFB). The transduction of the signal induces the ubiquitination of the AUXIN (Aux)/INDOLE-3-ACETIC ACID (IAA) proteins by the $SCF^{TIR1/AFB}$ complex and degradation by the 26S proteasome, releasing AUXIN RESPONSE FACTOR (ARF) proteins. ARF act as repressors or activators of transcription. The signal transduction downstream of Aux/IAA and ARF proteins activates or represses many auxin-induced responses, including cell wall loosening, cell elongation, cell cycle, hormone homeostasis and root meristem patterning. As the ARFs and the AUX/IAA are members of multi-genes families, the transcriptional effects of auxin depend on its concentration and the combinatorial expression of AUX/IAA and ARFs. The first committed step in crown root initiation is controlled by the CROWN ROOTLESS1 (CRL1)/ADVENTITIOUS ROOTLESS1 (ARL1) and CROWN ROOTLESS5 (CRL5), a plant-specific LATERAL BOUNDARIES DOMAIN (LBD) protein and an APETALA2(AP2)/ETHYLENE RESPONSIVE FACTOR (ERF), respectively. In rice, disrupting the *TRYPTOPHAN AMINOTRANSFERASE 1* (*TAA1*), which functions upstream of *YUCCA* genes in the auxin biosynthesis, reduces crown root development. The regulatory role of auxin in crown root initiation

has been further emphasized in studies of*arl1* and *crl1* mutant plants. The *arl1/crl1* mutants, devoid of crown roots, carry one primary root and fewer lateral roots and show an abnormal gravitropism response at the root level.

Cytokinins are antagonists to auxin. Whereas auxin stimulates root initiation but inhibits elongation, CKs inhibit initiation but support elongation. In cereals, the silencing of gene encoding proteins involved in the CK metabolism affects root development. In rice, the *hk5 hk6* double mutant with a defect in CK signalling presents a severely reduced root growth and an enlarged root cap. It is interesting to note that the crown rootless phenotype of *taa1* mutants was partially rescued by the overexpression of the *WUSCHEL-related Homeobox (WOX) 11* transcription factor. OsWOX11 is part of the CK signalling pathway and directly represses OsRR2, a type-A cytokinin-responsive gene. Interestingly, OsWOX11 is induced by both auxin and CK, affecting both auxin and cytokinin-responsive gene expression.

In flooding conditions, ethylene is produced and promotes crown root emergence at submerged nodes through induction of epidermal cell death. Ethylene stimulates auxin biosynthesis and basipetal auxin transport toward the elongation zone, where it activates a local auxin response leading to inhibition of cell elongation. In rice, the crown root primordia development requires the transcription factors *CRL5* and*CRL1* , both being ethylene- and an auxin-responsive gene. In rice, the 1-aminocyclopropane-1-carboxylic acid synthase (ACS), the rate-limiting enzyme in ethylene biosynthesis, is involved in the control of RSA. A stimulatory role for ethylene in lateral root development under Pi-deficient conditions has been suggested. Indeed*Osacs* mutants produce shorter roots and almost fail to promote lateral root growth in response to Pi deficiency.

In maize, ABA altered the polar localization of *ZmPIN1* , disrupted the distribution of auxin and inhibited lateral root initiation and development. In general, ABA has an antagonistic effect on lateral root primordial formation and emergence of auxin, which initiates lateral roots, finally affecting the size and architecture of the root system.

The role of strigolactones in root development has been demonstrated in several plant species. They promote crown root elongation and repress the formation of lateral roots. Rice *dwarf* mutants, impaired in strigolactone biosynthesis or signalling, showed shorter crown roots than the wild type. Exogenous application of GR24, a synthetic analogue of strigolactones, rescued the phenotype in biosynthetic mutants in a concentration-dependent fashion. On the other hand, signalling mutants were insensitive to it, proving that strigolactones – or their derivatives – have a direct function in crown root elongation. Lateral roots of *dwarf* mutants did not present a higher density than the wild-type one, contrary to what was observed in Arabidopsis strigolactone mutants. In both cases, though, treatment with exogenous GR24 decreased the density in wild-type and SL-deficient plants but not in strigolactone-insensitive mutants. The interaction between strigolactones and auxin seems to be crucial for lateral root development. Strigolactones probably inhibit lateral root formation by reducing PIN protein levels.

4.2. Hormones in root elongation

A functional root apical meristem ensures proper root elongation. The root meristem structure consists of three specific zones: the proximal meristem zone, including the quiescent centre responsible for maintaining the stem cell population in the root apical meristem, the transition zone and the elongation–differentiation zone. The root growth rate is highly correlated to the size of the root meristem, the latter being determined by a finely adjusted balance between cell division, expansion and differentiation. The antagonism between auxin and cytokinins contribute to the maintenance of the proper root meristem structure.

In recent years, evidence accumulated on ethylene's role in controlling root elongation at two different levels: regulating cell proliferation in the root apical meristem and cell elongation in the elongation zone. Strigolactones are also suggested to regulate primary root length. Indeed, exogenous application of GR24 led to elongation of the primary root and increased meristem cell number. In rice, the role of strigolactones on root elongation might be the control of cell division. In Arabidopsis, low ABA concentrations promote root growth by encouraging the quiescent centre and suppressing stem cell differentiation; in rice, under nonstressed conditions, ABA stimulates root hair elongation in a polar auxin transport-dependent manner.

The fact that ABA supports root elongation was also demonstrated in transgenic rice lines in which one of the ABA-catabolic enzymes was knocked out.

4.3. Hormones in root angle determination

Root growth angle determines whether the root will grow deep into the soil or opposite at the surface in the upper layers of the soil. The final root growth angle reflects three environmental responses: gravitropism, phototropism and hydrotropism. Roots show positive gravitropism. The ability to sense gravity is correlated with the presence of statoliths in the columella cells (forming the root cap). Statoliths are amyloplasts that sediment at the bottom of the columella cells. When gravity orientation changes by rotating plants, the statoliths start falling to the new bottom side of the cells. It is assumed that this movement distorts the endoplasmic reticulum, releasing Ca^{2+} into the cytoplasm and consequently modifying the intracellular pH. Changes in pH might affect polar auxin transport while inducing the re-localization of PIN proteins. This results in an unequal distribution of auxin from columella cells to the lateral root cap and auxin transport from the root cap to the epidermal cells of the elongation zone. This leads to differential cell elongation and root curvature in the elongation zone. PIN proteins and the auxin influx carrier AUXIN RESISTANT 1 (AUX1) have been reported to function in root gravitropism. It is fascinating to note that the root angle varies between the different classes of roots (primary, lateral, seminal). It has been proposed that this behaviour reduce self-competition and maximise the soil volume to be explored.

Root growth angle does not solely rely on response to gravitropism but also nutrient availability, temperature or hydropatterning. For this developmental process, literature exclusively mentions auxin's role while other hormones are not reported. This is understandable as all mutants affected in root angle growth are related to the auxin signalling pathway. Almost 20 years ago, Aloni and coworkers suggested for the first time that cytokinins control the early response to gravitropism. Indeed, a gravistimulation induced an asymmetric accumulation of cytokinin in the statoliths within less than 30 min. This undoubtedly caused the downward curvature near the root apex. In Arabidopsis, cytokinins could interact with auxin, ethylene and glucose signalling to trigger directional root growth. Whether such interactions are conserved in cereal crops is unknown. Interestingly, mutant loss-of-function in *CRL4/OsGNOM1* , *CRL1* or *CRL5*genes affected crown root development and gravitropic response. To understand the genetic control of root system architecture, including root growth angle, several QTLs and genes have been determined to play a central role in root growth angle in cereals.

5. CRISPR to modify the root system architecture

Genome editing – also known as gene editing – can be defined as a set of methods that enables targeted genome alterations. Over the past few years, this field has rapidly developed and proved promising in various areas, including basic biomedical research, medicine, and applied biotechnology. Three central platforms based on sequence-specific nucleases (SSNs) are currently in use: zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and RNA-guided CRISPR (cluster of regularly interspaced palindromic repeats)–Cas (CRISPR-associated protein) nucleases (RGENs).

Sequence-specific nucleases operate by inducing double-strand breaks (DSBs) in site-specific chromosomal positions. The cellular DNA repair mechanism is stimulated as soon as a double-strand break occurs, activating two main groups of repair pathways: either one that needs a homologous sequence as a template (homology-directed repair, HDR) or one that has no or little requirement for sequence homology (non-homologous end-joining, NHEJ, and microhomology-mediated end-joining, MMEJ, respectively). The homology-directed repair relies on copying the target-homologous donor template to achieve accurate gene/sequence replacement. The NHEJ pathway is error-prone and often leads to nucleotide insertions and/or deletions (INDELs) and substitutions. MMEJ can, in the presence of a target-specific repair DNA, introduce insertions or otherwise produce specific deletions.

Methods using ZFNs and TALENs, first-generation genome editing tools, were first published in 1996 and 2010, respectively. Both involve artificial fusion proteins consisting of a sequence-specific DNA-binding domain (DBD) fused to the non-specific DNA cleavage domain of the restriction endonuclease *Fok* I. These

two technologies have been employed to alter endogenous genes in more than forty organisms, even though protein design hinders their broader adoption.

Only three years after the TALE recognition code had been deciphered, the CRISPR-Cas9 technology entered the scene, revolutionising biological research. This gene-editing tool originated from the type II CRISPR-Cas system, an adaptive immunity system of bacteria and archaea that protects against viruses and plasmids. Its power lies in its simplicity: only two components, namely a single guide RNA (sgRNA) and the Cas9 endonuclease are in principle required to target any DNA sequence in any organism.

The defence process integrates short segments of invading DNA (called "spacers") into the bacterial genome, between copies of identical repeats; the resulting locus has been named CRISPR. Upon successive invasions, the CRISPR array is transcribed as precursor CRISPR RNA (pre-crRNA), processed to form a mature crRNA specific to the target sequence. The trans-activating crRNA (tracrRNA), target-independent, is also transcribed and promotes pre-crRNA maturation. The tracrRNA pairs with the repeat sequence of the crRNA and activate the Cas9 endonuclease, guiding it to the target sequence of the pathogen (named "protospacer"). A prerequisite for cleavage is the presence of the protospacer adjacent motif (PAM), a 2-6 base pairs sequence downstream of the target, which discriminates between "self" and "non-self" DNA possible, thus avoiding autoimmunity. PAM recognition by Cas9 initiates DNA unwinding and base pairing between crRNA and protospacer, prompting Cas9 to generate a DSB three base pairs upstream of the PAM.

In 2012, Jinek and colleagues demonstrated that changing 20 nucleotides in the crRNA made it possible to reprogram the target DNA sequence. The crRNA:tracrRNA complex could be engineered in a chimeric single guide RNA (sgRNA).

These findings enabled the transition of CRISPR-Cas9 from a biological system to a two-component genome editing tool: as mentioned above, it only consists of the Cas9 enzyme, which cleaves the DNA three base pairs upstream the PAM, and a sgRNA, that guides the nuclease to the target sequence. To overcome the target site limitation depending on PAM, several Cas variants from different organisms and after targeted evolution had been described and used in several plant species in recent years. Tools for the base and prime editing without double strand-break induction were developed. These systems allow the defined replacement of single nucleotides or short stretches of a genomic sequence.

Compared to first-generation platforms, the CRISPR-Cas9 technology offers many significant improvements. For example, while ZFNs and TALENs require the reengineering of the nuclease for each target sequence, the Cas9 protein is identical for all applications, so it is possible to address any genomic target by changing the sequence of the sgRNA following Watson-Crick base-pairing rules. Furthermore, designing and engineering guide RNAs is relatively simple, fast and low-priced, in contrast to the laborious and more expensive process demanded ZFNs and TALENs production. Another advantage of the CRISPR-Cas9 system is the possibility of using multiple sgRNAs with different sequences to target more than one locus simultaneously.

6. Use of CRISPR to study phytohormone-regulated root development

CRISPR technology offers great potential to study how individual genes dictate plants' developmental or adaptive processes (Table 1). Great effort has been made to understand the role of individual phytohormones in the developmental processes, but many questions remain to be answered in the future. In recent years, CRISPR-Cas technology accelerated the research on the developmental processes in rice, also focusing on many targets involved in phytohormone-mediated regulation. Here, we give examples of how CRISPR technology can help elucidate the phytohormone-regulated root developmental processes.

Auxin signalling pathway

Auxin is believed to be the main regulator of root development in plants. As such, many components of auxin biosynthesis, transport, or signalling become targets of genome editing. A high number of members represents the individual gene families involved in auxin signalling pathways and auxin transport. Whole-genome identification studies identified 31 *AUX/IAA* gene family members, 25 *ARFs* , 5*AUX1/LIKE AUX1* (*LAX*) and 12 *PINs* in rice. Members of these gene families frequently possess redundant functions. To

understand the contributions of individual members to the resulting phenotype, a comparison of single mutants and higher-order mutants is often necessary. CRISPR-Cas tools brought new opportunities to study these multigene families. With the CRISPR-Cas system, the production of higher-order knockout mutants is faster and more precise. Li *et al* . employed this strategy to analyse the *PIN* gene family in rice. Single and double mutants of four paralogous *OsPIN1* genes were obtained by multiplex CRISPR-Cas9 system. Due to functional redundancy, single *pin1* mutants do not show profound differences in the root phenotype compared to the control plants. Only the double mutant *pin1a pin1b* shows reduced primary root length, crown root and lateral root numbers, as well as reduced gravitropic responses, suggesting overlapping roles of *PIN1a* and *PIN1b* genes in auxin-regulated root development.

Similarly, five members of the rice OsTIR1/AFB auxin coreceptor family were studied to understand whether they have similar or diversified functions during plant development. Mutants in *OsTIR1/AFB* genes were obtained by CRISPR-Cas9 technology. In this case, two gRNAs were designed for each member of the *OsTIR1/AFB* family, one of which was specifically targeting the F-box coding region. In agreement with the findings from TIR1/AFB family in Arabidopsis, rice *OsTIR1/AFB*homologous genes also possess partially overlapping functions in diverse developmental processes. Analysis of single mutants and a double*Ostir1 Osafb2* mutant revealed OsTIR1 and OsAFB2 as crucial mediators of the auxin signal, with only a minor prevalence of OsTIR1 in the development of primary root and adventitious roots.

In rice, OsAUX1/LAX represent another class of carriers involved in polar auxin transport. Before the CRISPR-Cas system started to be commonly used in rice research, only one member of the OsAUX1/LAX family, OsAUX1, was functionally characterized employing T-DNA insertional mutagenesis, RNAi and over-expression. Later, two other auxin carrier proteins, OsAUX3 and OsAUX4, were analyzed using the CRISPR-Cas system. Two independent mutant lines were produced for both OsAUX3 and OsAUX4 carriers. When targeting *OsAUX3* , the capacity of CRISPR-Cas9 to create different types of mutations was used. Two gRNAs were designed to remove a protein domain precisely, which is unique among the OsAUX1/LAX protein family members. Interestingly, the phenotype of the *Osaux3-1* mutant, lacking the specific domain, was similar to the phenotype of the *Osaux3-2* null mutant, suggesting an essential role of this protein domain in determining the function of OsAUX3. Opposite to OsAUX1, OsAUX3 and OsAUX4 positively regulate primary root elongation while negatively affecting root hair length.

As well as with other auxin signalling components, studying the multigene AUX/IAA family can be relatively challenging. Surprisingly, Jun *et al.* revealed the crucial role of OsIAA23 in root development. An EMS-induced point mutation in the core region of domain II of OsIAA23 protein resulted in a severe pleiotropic root phenotype caused by the defect in quiescent centre maintenance. Therefore, to reduce the severity of the phenotype, Jiang *et al* . decided to produce different allelic versions of *OsIAA23* while preserving the core region of OsIAA23 domain II. To achieve that, CRISPR-Cas9 gRNA was designed to obtain mutations just next to the core region of domain II. Indeed, the *Osiaa23* mutants still showed an extensive reduction in lateral root number, but the overall effect on the phenotype was reduced. Besides, this study demonstrates that studying protein motifs and the function of specific protein domains with CRISPR-Cas becomes more straightforward than before.

Cytokinin signalling pathway

It is generally accepted that auxin and cytokinin cooperate in dictating the root developmental signals, but their phytohormone crosstalk's mechanism remains only partially understood. Apart from the modulation of auxin signals, cytokinin signalling controls many developmental processes of the root system. While the role of cytokinins in root development in Arabidopsis was already well studied, the molecular mechanisms involved in cytokinin signalling and its implication in cereal root system development are still not fully understood. Genome editing has already been utilized in the study of different components of cytokinin signalling or metabolism. One of the first genome editing targets involved in cytokinin regulation was *HvCKX1* in barley. The knockout of *HvCKX1* exhibited inhibition of the root system in 2 weeks old seedlings.

In contrast, Gasparis *et al* . observed increased total root length, fresh weight and total surface area of

10-day-old seedlings of the barley *ckx1* knockout line, which was characterized by decreased CKX activity. The opposite effect on the root system parameters was observed in the *ckx3* line, which showed increased CKX activity. A follow-up study would be necessary to clarify the role of individual *CKX* genes in barley root system development.

To study the cytokinin histidine kinase receptors in rice, gRNA sequences specific for both *OsHK* genes were used in tandem to produce the *hk5 hk6* double mutant. Assessing the phenotype of the single mutants, only *hk6* showed a slight increase in the primary root length and the number of lateral roots compared to the wild type. Oppositely, the double *hk5 hk6* mutant displayed severe pleiotropic defects in both roots and shoot parts.

Genome editing was employed to specifically knockout members of a class of cytokinin signal regulators, type B response regulators (RR). In this case, the CRISPR-Cas9 cassette consisted of a tandem array of four unique gRNA sequences targeting *RR21* , *RR22* , *RR23* , and *RR24* genes in rice. In this way, a triple mutant *rr21 rr22 rr23* was isolated and analyzed. Apart from the defects associated with floral development, the triple mutant also had shorter seminal roots and decreased lateral root density, likely linked to reduced cell proliferation.

CRISPR represents a handy tool that allows us to broaden our knowledge of the different regulatory components involved in root development and bring us closer to understanding the complexity of the underlying root development mechanisms.

7. Enhancing tolerance to abiotic stress conditions by CRISPR-Cas-induced modulation ofroot system architecture

Modifying specific root system architecture characteristics proved beneficial to enhance tolerance to unfavourable conditions of the environment. Interestingly, the most recent genome editing strategies for changing the cereal root system architecture alter the growth angle of the roots. Kitomi *et al* . introduced a promising approach to enhancing salinity tolerance by modulating root system architecture in rice. In high saline conditions, the soil surface root phenotype was proposed to offer plants the possibility of avoiding unfavourable conditions. The loss-of-function allele of *DEEPER ROOTING1*(*DRO1*)-*like* (*DRL1*) was identified and shown to be responsible for the soil surface root phenotype in rice while being negatively regulated by auxin. Analysis of CRISPR-Cas9 mutants revealed that *DRO1, DRL1,* and another *DRO1* -homolog, *DRL2* , control the root growth angle in rice, with *DRO1* and *DRL1*being more important in the gravitropic response. More recently, a mutant with a steep seminal root and lateral root growth angle was discovered in a mutagenized population of barley. The steeper root system architecture can potentially be more advantageous in drought conditions. It was shown that the root angle is regulated by*ENHANCED GRAVITROPISM2* (*EGT2*) gene, which is conserved in barley and wheat, and that *EGT2* acts in an auxin-independent manner.

Moreover, the authors suggest that more genes with function in the regulation of root growth angle could be identified in the future. As*DRO1* -family genes and the *EGT2* gene have a specified role in RGA, without adverse effect on other morphological traits, they could be very promising genome editing targets for improving the tolerance to various abiotic stress factors. It would be interesting to transfer the knowledge to other cereal crops and to test their response to different stress factors.

While auxin and cytokinin predominantly control the developmental processes, other plant hormones play essential roles in regulating the plant ability to adapt to a changing environment. Abscisic acid responses are generally involved in drought stress. It was shown that targeting genes involved in ABA catabolism are a promising strategy to enhance tolerance to abiotic stress conditions. CRISPR-Cas9 mediated knockout of rice *ABA 8'hydroxylase* (*OsABA8ox2*), an enzyme involved in ABA catabolism, resulted in elevated ABA and IAA levels in rice. Changes in the phytohormone status were reflected in the high root-to-shoot ratio and lateral root density under drought stress conditions, leading to an increased survival rate after drought stress and subsequent rewatering. Another study focused on one of the factors regulating ABA responses, *ENHANCED RESPONSE TO ABA1*(*ERA1*), which is encoding β-subunit of the protein farnesyltransferase. The role of this posttranslational modification enzyme was partially revealed by analysis of CRISPR-Cas9

mutants. Three gRNA sequences targeting three different exons of *OsERA1* were separately cloned in a binary vector and used for rice transformation. Homozygous mutations in two of these gRNA target sites in the*OsERA1* gene were lethal already in early development. Only mutants with 1-bp insertion in the first exon of *OsERA1* could be further analyzed. *Osera1* mutants exhibited increased primary root growth under nonstressed conditions and enhanced drought responses, resulting from the increased sensitivity to ABA. These findings imply that the natural ability of ABA to modulate root system architecture could be utilized in novel strategies for improving drought tolerance in cereal plants.

8. Future and perspectives

Understanding how plants adapt their root system under changing environmental conditions to ensure the continued uptake of water and nutrients is essential for future breeding programs. Due to the increased occurrence of weather extremes, crops will need to be adapted more quickly in the future. Previous classical methods, such as cross-breeding and selection, are not sufficient for this purpose. CRISPR/Cas technology has proven to be a tool that allows precisely targeted modification of the target sequence. By refining the methods (base editing, prime editing) or tissue-specific expression of the double-strand break-inducing agents, the often pleiotropic effect of different mutations can be circumvented. The examples shown in the table can also be applied to other crops. For instance, it would be interesting to see if the *DRO1* gene studied in rice plays a role in other cereals' salt and drought stress tolerance. Since knockout of the *CKX* genes in barley did not lead to the results obtained in maize, tissue-specific expression of the molecular scissors could be used as an alternative to circumvent adverse pleiotropic effects. Furthermore, Cas fusion proteins show real potential for activating or repressing the mentioned genes.

Molecular mechanisms of hormonal influence on root growth have relevance for the plant's abiotic and biotic interaction. This requires knowledge of the metabolism and signalling pathways involved. Also, specificity of molecular regulation exists among cereals, depending on their growth habitat. Although some futures have been conserved during evolution, there are variations between the dicot model plants to cereals. Therefore, interest should also be given to more minor or non-cereal plants.

Because targeting the hormone metabolism and signalling pathway has very often adverse pleiotropic effects, one should consider focusing on the downstream events specific to RSA regulation. For example, targeting the root angle would have extreme advantages for the plant in periods of temporary drought. At the same time, mechanical anchoring of the plant would be improved, which could be advantageous during strong winds or thunderstorms. It is also interesting to improve the nutrient uptake of the root by soil microbes. By targeting secreted signals, the microbial community surrounding the root can be influenced.

In summary, CRISPR/Cas technology with its multiple variants and fusion possibilities allow for an incredible variety of modifications. In the future, this will enable a more targeted and rapid transfer of primary research results into current breeding programs.

9. Acknowledgements

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10. Conflict of interest

The authors declare to have no conflict of interest.

11. References

Table 1. Examples of CRISPR-Cas-mediated approaches used in the study of root development.

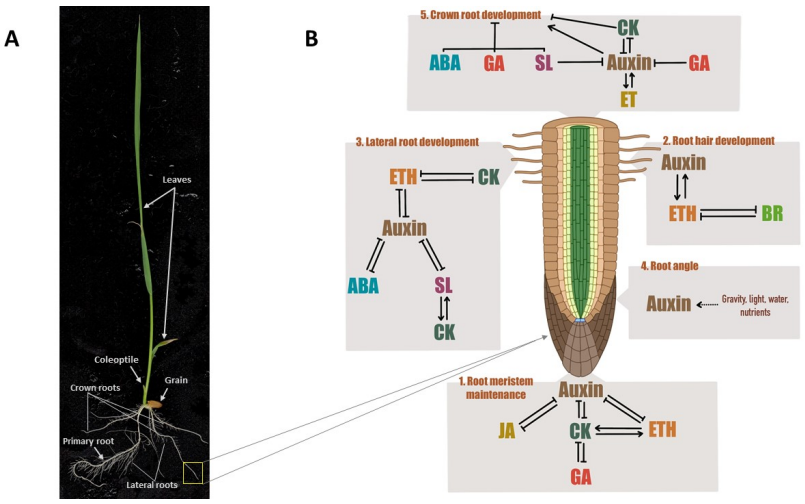
Target gene	Crop	Promoter::Cas variant	Root-associated parameters	References
Involved in auxin-mediated root growth	**Involved in auxin-mediated root growth**	**Involved in auxin-mediated root growth**	**Involved in auxin-mediated root growth**	**Involved in auxin-mediated root growth**
OsPIN1a-OsPIN1d	rice	*Cas9* (promoter not specified)	primary root length, crown root number, lateral root number, CRP number, root growth angle	
OsAUX3	rice	*OsUbi::Cas9*	lateral root number, primary root length, root hair length	
OsAUX4	rice	*OsUbi::Cas9*	primary root length, root hair length	
OsTIR1, *OsAFB2*, *OsAFB3*, *OsAFB4*, *OsAFB5*	rice	*OsUbi::Cas9*	primary root length, crown root number, lateral root number	
OsIAA23	rice	*ZmUbi::Cas9*	lateral root number, crown root number, primary root length, crown root length, total root weight	
OsNAC2	rice	*35S::Cas9*	primary root length, crown root number	
TAA1/FIB	rice	*ZmUbi::Cas9*	crown root number, lateral root number	
DRO1, *DRL1*, *DRL2*	rice	*2x35S::Cas9*	root growth angle	
Involved in cytokinin-mediated root growth	**Involved in cytokinin-mediated root growth**	**Involved in cytokinin-mediated root growth**	**Involved in cytokinin-mediated root growth**	**Involved in cytokinin-mediated root growth**
HK5, *HK6*	rice	*ZmUbi::Cas9*	primary root length, lateral root number	
RR21, *RR22*, *RR23*, *RR24*	rice	*ZmUbi10::Cas9*	seminal root length, lateral root number	
CKX1	barley	*ZmUbi::Cas9*	total root length, total surface area, dry weight	

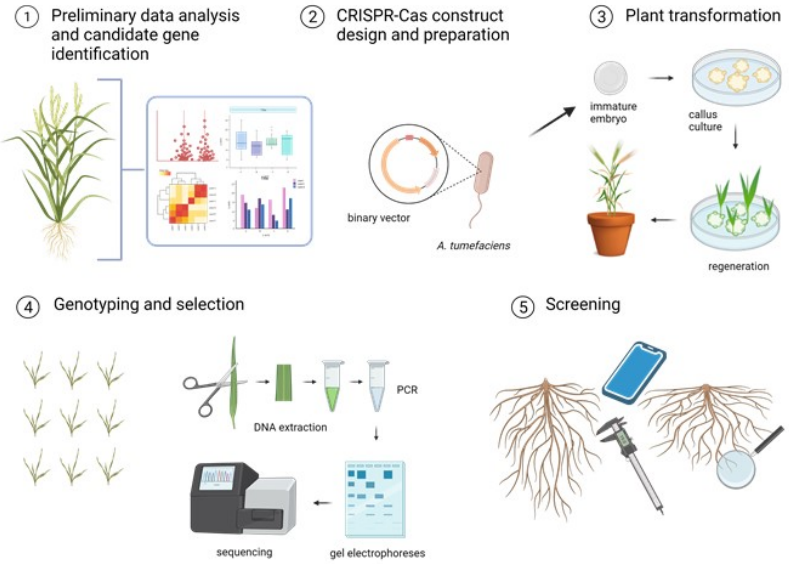
CKX1, *CKX3*	barley	*ZmUbi::Cas9*	total root length, total surface area, root mass, root diameter	
Involved in abscisic acid-mediated root growth	**Involved in abscisic acid-mediated root growth**	**Involved in abscisic acid-mediated root growth**	**Involved in abscisic acid-mediated root growth**	**Involved in abscisic acid-mediated root growth**
OsABA8ox2	rice	*Ubi::Cas9*	root elongation, root-to-shoot ratio	
OsERA1	rice	*2x35S::Cas9*	primary root length	
Involved in ethylene-mediated root growth	**Involved in ethylene-mediated root growth**	**Involved in ethylene-mediated root growth**	**Involved in ethylene-mediated root growth**	**Involved in ethylene-mediated root growth**
OsACS1, *OsACS2*	rice	*Ubi::Cas9*	lateral root elongation in response to Pi deficiency	
not specified	**not specified**	**not specified**	**not specified**	**not specified**
OsRopGEF3	rice	*OsUbi::Cas9*	root hair length and width	
OsLPR5	rice	*ZmUbi::Cas9*	primary root length	
EGT2	barley	*ZmUbi::SpCas9*	seminal root angle, lateral root angle	

12. Figure legends

Figure 1. Picture of the root system of an 11 days-old rice seedling (A) and schematic overview of phytohormonal regulation of root system architecture (B). ABA – abscisic acid, CK – cytokinin, ETH – ethylene, GA – gibberellic acid, BR – brassinosteroids, JA – jasmonic acid, SL – strigolactone.

Figure 2. Workflow for CRISPR-mediated knockout of genes involved in root system architecture.





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